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(57) Abstract

A method for identifying bacteria in a sample is described which comprises amplifying a portion of the 23S rDNA present in the sample using, as one primer, a degenerate primer set comprising one or more DNA molecules consisting essentially of DNA having the sequence(s) 5'GCGATTTCYGAAYGGGGRAACCC, the other primer consisting of DNA having the sequence 5'TTCGCCTTTCCCTCACGGTACT and testing the resulting amplicon by hybridisation to one or more oligonucleotide probes designed to identify one or more bacteria likely to be present in the sample. The method allows for the identification of at least 8 and considerably more bacterial species in a single test, including Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus spp., Klebsiella spp., Enterobacter spp., Proteus spp, Pneumococci, and coagulase negative Staphylococci. One or more novel oligonucleotides for use in this test are immobilised on a solid carrier and incorporated in a diagnostic test kit for use in hospitals and other environments.

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IDENTIFICATION OF BACTERIA

This invention relates to the identification of bacteria and more particularly, although not exclusively, to the identification of clinically important bacteria in biological samples e.g. blood. The invention is of special application to the identification of clinically important bacteria isolated in a hospital laboratory and obtained directly from clinical specimens, including positive blood culture bottles and fresh blood specimens. For convenience, the invention will be described primarily in the context of clinical needs but it will be appreciated that it has wide application outside this field.

Eight bacterial species account for 65% of all blood culture isolates, although this varies with patient population. Typically these are Escherichia coli (~20%), Staphylococcus aureus (~20%), Pseudomonas aeruginosa (7%), Enterococcus spp. (5%), Klebsiella spp. (~5%), Enterobacter spp. (~4%), Proteus spp, and Pneumococci (~3%). In addition coagulase negative Staphylococci are frequently isolated from patients with intra-vascular devices but many of these isolates are clinically insignificant. The remaining 35% of blood culture isolates comprise upwards of 50 different species. Rapid detection of these numerous species with a single test would be very useful.

In recent years much effort has been invested in the production of species specific primers which can be used to identify an organism in a simple PCR reaction. If a PCR product of the expected size is produced with a set of these primers the presence of the target bacterium can be predicted with almost total certainty. Unfortunately this approach is not ideal for analyzing samples which may contain one of many pathogens. Analysis of such specimens using this approach requires a multiplex PCR containing a complex mixture of primers, a series of individual PCR reactions run in parallel to detect each species which may be present, or a series of PCR reactions run sequentially. Because of the potentially large number of different bacterial species that may be isolated from blood, these methods are unsatisfactory for the routine screening of general microbiological specimens.

A better approach is to use a single pair of primers to amplify DNA from a variety of organisms and then to analyze the sequence of the resulting product to determine from which species it originated. Primers directed at conserved stretches of DNA will produce an amplicon e.g. a PCR product from almost all species of bacteria. The region usually chosen is the 16S rDNA or the 16S 23S rDNA spacer region. The 16S 23S rDNA spacer region is highly variable within many species, frequently containing tRNA genes, and the length and sequence of amplified products can be used to type strains within a single species. In contrast the 16s rDNA is highly conserved and, as a large amount of sequence data is available on public computer databases, sequence data can give a definitive identification of the species of a bacterium in many cases. Unfortunately some species of clinical significance have identical or very similar 16s rDNA sequences which would be impossible or difficult to discriminate using this region alone.

We have now found that by targeting the large ribosomal sub-unit (23s rDNA) with novel specially designed oligonucleotide primers, specified hereinafter, and amplifying a portion of this DNA we can identify a large number of bacteria by means of a single test or at most a very small number of tests. For convenience, amplification by means of the polymerase chain reaction (PCR) will be referred to throughout the following description. It will be appreciated, however, that any other amplification technique can alternatively be used e.g. transcription mediated amplification (TMA), reverse transcriptase polymerase chain reaction (RTPCR), Q-beta replicase amplification, and single strand displacement amplification. Some modification of the primers used for PCR may be necessary when using these alternative methods. In the case of the TMA method, such modification will usually require the addition of promoter and recognition sequences to the primers of the present invention.

In accordance with the present invention the bacterial species are detected by amplifying bacterial 23S rDNA, and identified by using the amplified product (amplicon) to probe one or more oligonucleotides in a reverse hybridization system. After amplification by universal primers, the sequence of the amplicon has to be determined. Direct sequencing is complex and expensive. Sequence variation can be identified by restriction digests, but

this is not a practical way to detect a wide range of variants. According to this invention the labelled amplicon is preferably hybridized to a panel or an array of oligonucleotides immobilized on a solid phase such as, for example, nylon membranes or synthesized in situ on silicon wafers. Since both the target and the probe are present at much higher concentrations than is typical for a Southern blot these hybridization reactions can be carried out in very short periods of time (less than 1 hour). This method is referred to as reverse hybridization. Reverse hybridization allows a very large series of sequence variations to be positively identified and lends itself to automation.

The present invention comprises primers that amplify a portion of the 23S rDNA. The DNA sequences of these primers are set out below.

Sequence 5' to 3'

Forward primer ST23SP6
SEQ ID No 1 GCGATTTCYGAAYGGGGRAACCC

Reverse Primer ST23SP10
SEQ ID No 2 TTCGCCTTTCCCTCACGGTACT

The sequences of the primers and oligonucleotides are given herein and expressed in standard IUB/IUPAC nucleic acid code. The primers, especially the reverse primer, are appropriately labelled e.g with Digoxigenin (as in the Example given below), biotin, or fluorescein. Any other labelling system can be used. Hybridization can also be detected by using the oligonucleotides to construct molecular beacons.

The Forward primer sequence given above contains the symbols Y and R. In accordance with standard terminology for use with degenerate sequences, Y represents nucleotides C or T and R represents nucleotides A or G. The symbols Y and R are used to indicate variability of base permutations at "wobble" regions in the sequence. The Forward primer reagent is therefore prepared as a degenerate primer set using a mixture of the appropriate nucleotides for incorporation at the wobble points.

The PCR products produced by these primers, from a range of medically important Gram positive and Gram negative bacterial cultures, are characterized by hybridization to an array of oligonucleotides designed to identify taxonomic groups. Using this procedure, which takes typically less than four hours, we have been able to identify a wide range of genera and species. This approach allows bacteria and mixtures of bacteria to be identified by molecular methods without the need for a priori knowledge of the causative agent or agents.

The oligonucleotide probes, the sequences of which are set out below, can be used singly for the identification of certain individual species or in a panel or array for the identification of many different species. There is theoretically no limit to the number of oligonucleotide targets employed and the number of species that can be identified.

Ideally the oligonucleotides used should hybridize only to one bacterial species and to all members of that species. Thus with an ideal array, a unique profile consisting of species specific spots would be seen, giving identification to the species level. In practice, two or more oligonucleotide spots may be required for many species and in some cases several such spots may allow identification of variation within a species. In addition, some identifications can be made by comparing the relative intensities of hybridization of individual species to individual oligonucleotides. The assessment of hybridization can be quantified by visual or automated methods.

For example, 27 oligonucleotides have been used for the unambiguous identification of Pseudomonas aeruginosa, Proteus mirabilis, Enterococcus feacium and Enterococcus feacalis , as well as Staphylococcus aureus, coagulase negative Staphylococcus, Listeria species, Stenotrophomonas maltophilia , Burkholderia cepacia, and Escherichia coli. Usually, therefore, it will be desirable to provide oligonucleotides to probe not only for the 8, 10, or more of the micro-organisms commonly occurring in hospital samples or the samples being tested in other situations, but also for other organisms likely to be encountered. Preferably, probes for at least 30 different species of micro-organism will be present on the support substrate used in the test.

The detection of short sequences in amplified DNA is a straightforward procedure that can be carried out on a massively parallel scale. This may be achieved by hybridizing a labelled PCR product to an array of oligonucleotides immobilized on a solid support e.g. a membrane, glass slides, or microtitre trays, or synthesized in situ on silicon wafers.

This assay can be easily extended to identify a wider range of bacterial species with the addition of oligonucleotides without increasing the complexity of performing the assay.

The oligonucleotides are:

Oligo	Primary Target organism	Sequence 5' to 3'
1a	Proteus mirabilis	
SEQ ID No 3		AATAGCAGTGTCAGGAGAACGGTCT
1b	Proteus mirabilis	
SEQ ID No 4		ATAGCCCCGTATCTGAAGATGCT
1c	Escherichia coli	
SEQ ID No 5		CCAGAGCCTGAATCAGTGTGT
2a	Klebsiella oxytoca	
SEQ ID No 6		TCCCGTACACTAAAACGCACAGG
2b	Klebsiella pneumoniae	
SEQ ID No 7		TCCCGTACACCAAAATGCACAGG
2c	Escherichia coli	
SEQ ID No 8		CAGAGCCTGAATCAGTATGTG
3a	Enterobacter cloacae	
SEQ ID No 9		TCCCGTACACGAAAATGCACAGG
3b	Esh.coli, Citrobacter spp.	
SEQ ID No 10		CCCGTACACAAAAATGCACA

3c Salmonella enterica

SEQ ID No 11 AGAGCCTGAATCAGCATGTGT

4a Streptococcus spp. A

SEQ ID No 12 AGAAGAATGATTTGGGAAGATC

4b Pseudomonas aeruginosa

SEQ ID No 13 GCTTCATTGATTTTAGCGGAAC

4c Haemophilus influenzae

SEQ ID No 14 GTGAGGAGAATGTGTTGGGAAG

5a Streptococcus spp. B

SEQ ID No 15 AGAAGAAGACCTTGGGAAAGG

5b Enterococcus faecalis

SEQ ID No 16 GGTAGTCTGTTAGTATAGTTGAAG

5c Aeromonas hydrophilia

SEQ ID No 17 TGGAACGGTCCTGGAAAGGC

6a Streptococcus spp. B

SEQ ID No 18 AGAAGAACTACCTGGAAGGT

6b Enterococcus faecium

SEQ ID N 19 GGTAGTTCTTTCAGATAGTCGG

6c Staphylococcus warneri

SEQ ID No 20 ACGGAGTTACAAAAGTATATATTAGTTTTT

7a Staphylococcus aureus

SEQ ID No 21 ACGGAGTTACAAAGGACGACATTA

7b Staphylococcus spp.(+Listeria spp.)

SEQ ID No 22 GGTTGTAGGACACTCTATACGGAGTT

7c Staphylococcus saprophticus

SEQ ID No 23 ACGGAGTTACAAAAGAACAGACTAGTTTTT

8a Staphylococcus epidermidis

SEQ ID No 24 ACGGAGTTACAAAAGAACATGTTAG

8b Staphylococcus carnosus

SEQ ID No 25 ATGGAGTTACAAAAGAATCGATTAG

8c Staphylococcus haemolyticus

SEQ ID No 26 ACGGAGTTACAAAGGAATATATTAGTTTTT

9a Burkholderia cepacia

SEQ ID No 27 CGTATTGTTAGCCGAACGCTCT

9b Stenotrophomonas maltophilia

SEQ ID No 28 AGCCCTGTATCTGAAAGGGCCA

9c Listeria spp.

SEQ ID No 29 ACGGAGTTACAAAAGAAAGTTATAATTTTT

10a Streptococcus oralis

SEQ ID No 30 AGAAGAATGATTTGGGAAGATC

10b Streptococcus anginosus

SEQ ID No 31 AGAAGAAGACCTTGGGAAAGG

10c Streptococcus thermophilus

SEQ ID No 32 AGAAGAACTACCTGGGAAGGT

Oligonucleotides for use in an extended array.

Oligo	Primary Target organism	Sequence 5' to 3'
31	Streptococcus spp.	
SEQ ID No 33		ACGGCAGAAGGGCAAACCGAATTTTTT
32	Streptococcus spp.	
SEQ ID No 34		GGCAGGAGGGCAAACCGAAGATTTTTT
33	Streptococcus spp.	
SEQ ID No 35		GGCAAGAGGGCAAACCGAAGATTTTTT
34	Acinetobacter spp.	
SEQ ID No 36		CGCTCTGGGAAGTGCGAACGTTTT
35	Escherichia coli	
SEQ ID No 37		GAAAGGCGCGCGATACAGGGTTTT
36	Enterobacter cloacae	
SEQ ID No 38		GAAAGTCCGACGGTACAGGGTTTT
37	CNS A	
SEQ ID No 39		ACGGAGTTACAAAAGAACATGTTAGTTTTT
38	CNS B	
SEQ ID No 40		ACGGAGTTACAAAAGAATTTGTTAGTTTTT
39	Plesiomonas shigelloides	
SEQ ID No 41		GTTAGTGGAACGGATTGGAA
40	Neisseria gonorrhoeae	
SEQ ID No 42		TGACCATAGCGGGTGACAGTCTTT
41	Neisseria meningitidis	

SEQ ID No 43 TGACCATAGTGGGTGACAGTCTTT

42 Campylobacter spp.

SEQ ID No 44 GTGAGTTTAGCAGAACATTCTG

43 Campylobacter lari

SEQ ID No 45 TAAGTAAGGTTAGTAGAACACTCT

44 Helicobacter pylori

SEQ ID No 46 CATCCAAGAGAACGCTTTAGCA

45 Ralstonia spp.

SEQ ID No 47 AATGGGATCAGCCTTGTACTCT

46 Esh. coli 3

SEQ ID No 48 TCTGGAAAGGCGCGCGATACA

47 Enterobacter 1

SEQ ID No 49 GTCTGGAAAGTCCGACGGTAC

48 Chlamydia pneumoniae

SEQ ID No 50 ACCATATTTTTAATATGGGGTTTTT

49 Chlamydia psittaci

SEQ ID No 51 CCACATTTTTAATGTGGGG

50 Chlamydia trachomatis

SEQ ID No 52 CCGAGCTGAAGAAGCGAGGGTTT

51 Coxiella burnetti

SEQ ID No 53 CCTTTCGAGGTTATGTATACTGAA

52 Rhodococcus erythropolis

SEQ ID No 54 GGTGTTGCATTCGTGGGGTTG

53 Rhodococcus fascians

SEQ ID No 55 GGGTTGCGTATGGAGGGTTG

54 Mycobacterium tuberculosis

SEQ ID No 56 GCGCTACCCGGCTGAGAGG

55 Mycobacterium avium

SEQ ID No 57 CTACCTGGCTGAGGGGTAGTC

56 Mycobacterium kansasii

SEQ ID No 58 GGACGATACGTCTCAGCTCTA

57 +ve Positive control

SEQ I No 59 TGACTGACCGATAGYGAACCAGTA

(40) Neisseria gonorrhoeae

SEQ ID No 60 TGACCATAGCGGGTGACAGTC

(41) Neisseria meningitidis

SEQ ID No 61 TGACCATAGTGGGTGACAGTC

(48) Chlamydia pneumoniae

SEQ ID No 62 ACCATATTTTTAATATGGGG

(50) Chlamydia trachomatis

SEQ ID No 63 CCGAGCTGAAGAAGCGAGGG

The sequences of the primers and oligonucleotide priobes are also given hereinafter as Sequence Listings in written form and supplied in computer readable form. The information recorded in computer readable form is identical to the written sequence listing.

METHODOLOGY

The methods we have used are described as follows:

Bacterial strains. The stored strains used are listed in Table 1. Organisms were stored in glycerol broth at -70° C.

Blood cultures. Blood cultures may be performed by using an enrichment technique e.g. the Vital® automated system (Bio Merioux, France). In this method up to 10 mL blood is placed in both anaerobic and aerobic Vital blood culture bottles. The bottles are then incubated in the Vital machine and continuously monitored for evidence of bacterial growth. When possible growth is identified, the bottle is removed from the incubator and a sample taken for Gram staining and subculture to agar plates. Over a period of 25 days an additional sample of 100 microlitres for DNA extraction was taken from 116 unselected positive blood culture bottles, as described below. The DNA assay was performed without knowledge of the patient details or the initial Gram stain result.

Extraction of bacterial DNA from pure bacterial cultures. Stored organisms were sub-cultured onto Columbia Blood Agar plates (Oxoid, UK). A single colony of overnight growth at 37°C was suspended in 100 microlitres of distilled water containing 1 microlitre of a 1 mg/ml solution of lysostaphin (Sigma Chemical Co. UK) and incubated at 37°C for 10 minutes. The tubes were then transferred to a thermo-cycler (Perkin-Elmer 2400 Gene amp PCR system) and heated to 95°C for 10 minutes. Finally they were spun at 13,000 rpm for 2 minutes in a micro-centrifuge and 1 ml of the supernatant used in the 23S PCR described below.

Extraction of bacterial DNA directly from Vital blood culture bottles. DNA was extracted from all positive blood culture bottles in a Class II safety cabinet using the following protocol. Two to four drops of the broth were transferred into 0.5 ml of sterile distilled water at the time of aspiration for Gram stain and subculture. The tubes were spun at 13,000 rpm in a micro-centrifuge for 2 minutes and the supernatant discarded. The pellet was re-suspended in 100 microlitres of distilled water containing 1 microlitre of a 1 mg/ml solution of lysostaphin (Sigma, UK) and incubated at 37°C for

20 minutes in a dry block (Scotlab, UK). The temperature was then raised to 95°C and the tubes incubated for a further 15 minutes. Finally the tubes were spun at 13,000 rpm for 2 minutes in a micro-centrifuge and 1 microlitre of the supernatant used in the 23S PCR described below.

Design of primers to amplify 23S bacterial rDNA.

Forward primer ST23SP6

5' GCGATTTCYGAAYGGGGRAACCC

Reverse primer ST23SP10

5'digoxigenin-TTCGCCTTTCCCTCACGGTACT

Primers were commercially synthesized (Amersham Pharmacia, Amersham, UK). A PCR master mix containing 1 x DnaZyme buffer (Flowgen, UK), 1 microMole Primer ST23SP6, 2 microMoles Primer SP23SP10, and 150 microMoles of each dNTP was made up in 5 ml quantities. Forty microlitre aliquots of the master mix were dispensed into 100 microlitre PCR tubes. When the DNA extracts were available 1 microlitre of the appropriate extract and 1 unit of DnaZyme DNA polymerase (Flowgen, UK) added to each tube. The PCR mixes were then subjected to 5 cycles of 95°C for 15 seconds, 55°C for 15 seconds plus 72°C for 15 seconds, followed by 25 cycles of 95°C for 15 seconds plus 65°C for 30 seconds. The presence of a PCR product was confirmed by agarose electrophoresis of 5 microlitres and visualized with ethidium bromide.

Sequence determination of primary pathogens and identification of potential reverse hybridization targets.

Where species information was not available, we sequenced PCR products from selected isolates in our organism collection. This was supplemented by sequence data from products that failed to hybridize with the early oligonucleotide arrays or gave erroneous identifications. All the oligonucleotides chosen were targeted at sequences within a variable region of the PCR product. Using this sequence information, a panel of oligonucleotides with similar calculated melting temperatures was designed.

These sequences were tested in arrays using amplicons generated from reference organisms. Oligonucleotides not ideal as probes in the array due to low hybridization intensity were modified by the addition of low numbers of thymine bases (<20) to the 3' end of an oligonucleotide during synthesis. These modifications increase hybridization intensity. Thus by adjusting the number of thymine bases this technique was used to equalise the hybridisation intensity of the array.

Using this technique oligonucleotides with hybridization properties suitable for incorporation into the array were produced. This allows oligonucleotides that would have been unsuitable for inclusion in the array due to low intensity of hybridisation to be included in the same easily interpretable array.

Production of the hybridization membranes.

One form of layout of target oligonucleotides is shown in Figure 1. Oligonucleotides were synthesised and 50 pg of each in 0.3 microlitres of water were spotted onto a specific position on a nylon membrane (MAGNA Micron Separations inc. MA, USA). A 3 mm grid was printed on the membrane with a bubble jet printer to allow the spots to be more accurately positioned. Strips were made in batches of 20. Once all the oligonucleotides had been applied the strips were dried and exposed to short wave UV in an Amplirad light box (Genetic Research Instruments, Essex, UK). The length of exposure was found to have a marked effect on the intensity of the resulting spots: with our UV illuminator 30 seconds was found to give the optimal spot intensity. After the oligonucleotides had been cross-linked to the membrane, any unbound oligonucleotides were removed by washing twice in 0.5 x SSC plus 0.1 % SDS for 2 minutes at 37 °C. The strips were dried and stored at room temperature ready for use.

Hybridization protocol.

The digoxigenin labeled 23S rDNA amplicons were hybridized to the oligonucleotide arrays using the following protocol. Each membrane was numbered and placed in a separate 2.5 ml screw-topped micro-centrifuge tube containing 0.5 ml of 5 x SSC plus, 0.1% N-laurylsarcosine, 0.02% SDS, and 1% blocking reagent (Boehringer Mannheim,

Germany). The digoxigenin PCR products were heated to 95°C in a thermal cycler and the appropriate PCR product added directly to each tube. The hybridization was continued for 45 minutes at 50°C with gentle agitation. The strips were then removed from the tubes washed four times in 25 ml 0.25 x SSC plus 0.1% SDS, for each 20 strips, at 37°C for 2 minutes. Any hybridization was detected using an anti-digoxigenin antibody conjugated to alkaline phosphatase amd detected colorimetrically (Boehringer Mannheim system). Color development was clearly visible between 15 minutes and 1 hour.

Assessment of the primers. The effectiveness of the primers was first assessed with DNA extracts from 79 stored bacterial isolates representing 28 species (Table 1). All the isolates tested produced products. A band of approximately 420 bp was produced with Gram positive bacteria and one of 390 bp for the Gram negative bacilli. Two isolates of Candida albicans were also processed using the same protocol but no PCR products were seen. No bands were seen in the DNA negative amplification controls.

Hybridizations from enrichment broths.

Over the course of the study samples from 408 culture positive Vitec bottles were subjected to PCR on the day they became positive.

The results obtained by the hybridization assay were compared to those subsequently obtained by conventional bacteriology (culture followed by phenotypic identification). Three hundred and fifty bottles (83.7%) produced correct identifications. These included nine (2.2%) in which mixed cultures were correctly identified. Mixtures identified included Pseudomonas aeruginosa plus Enterococcus faecalis, Pseudomonas aeruginosa plus Stenotrophomonas maltophilia, Staphylococcus aureus plus Enterococcus faecalis, CNS plus Pseudomonas aeruginosa and CNS plus Enterococcus faecium. Streptococcal DNA was identified in six bottles but no organisms subsequently grown, possibly indicating contamination of the enrichment bottles with streptococcal DNA. The remaining 43 (10.5%) bottles either contained no bacteria to which oligonucleotides were targeted or a PCR product was not obtained.

ASSAY PROTOCOL

SOLUTIONS NEEDED

(1) Polymerase Chain Reaction mixture:
Forward primer ST23SP6
Reverse primer ST23SP10

The PCR master mix was made up in 2.5 ml quantities containing all the ingredients for PCR except DNA polymerase. 12.5 microlitre each primer 1 microgram/microlitre (Pharmacia), 5 microlitre each dNTP 100 mM (Pharmacia), 250 microlitres 10 x DnaZyme buffer (Flowgen, Staffordshire, UK), 2.2 ml water. This mixture should then be dispensed in 45 microlitre aliquots into 200 microlitre reaction tubes and 1 unit (0.5 microlitre) of Taq polymerase (DnaZyme) added to the tubes just before they are required.

(2) Maleic acid buffer pH (7.5): 4.13 g sodium chloride and 5.53g maleic acid in 500 ml of water, pH with 5 M NaOH

(3) Detection buffer pH (9.5): 6.05g tris-base and 2.97g NaCl in 500 ml of water, pH with 10 N HCl

(4) Blocking solution: 0.1 g Boehringer Mannheim blocking solution in 5 ml of detection buffer: make 2 hours before required.

(5) SSC: (20x) 3 M NaCl plus 0.3 M sodium citrate. Dilute to 0.25 x SSC and keep at 37°C ready for use.

(6) BCIP: 50 mg/ml 5-bromo-4-cloro-3-indolyl phosphate toluidinium salt in 100% dimethylformamide

(7) NBT: 75 mg/ml nitroblue tetrazolium salt in 70% dimethylformamide

METHOD

This procedure will identify bacteria from positive Vitec blood culture bottles (Bio-Merioux, France). When aspirating the broth for Gram staining and sub-culture add 2 to 4 drops of the positive Vitec broth to one of the 2 ml screw-capped tubes containing 0.5 ml of sterile water and label the tube with the lab number.

DNA extraction (To be carried out in the containment level 3 laboratory)

(1) Spin the screw-capped tubes at high speed (10,000g) for 4 minutes in a sealed rotor centrifuge.
(2) In a class 1 hood open the rotor and tubes and discard the Supernatant.
(3) Add 100 microlitres of a 1 microgram/ml solution of lysostaphin (Sigma UK) made up in water.
(4) Place the tubes in a covered dry block and incubate at 37° C for 20 minutes.
(5) Turn the dry block up to 95° C and leave for 15 minutes.
The PCR and hybridization may now be carried out on the open bench in a laboratory.

Preparation of the hybridization strips

Strips were made either using the VP-scientific (San Diego, CA, USA) multi print system which allows 96 spots to be simultaneously printed from a 384 well microtitre plate according to the manufactures instructions (replacing steps 1,2, and 3 below) or manually using the following procedure:

Manual production of hybridization strips

(1) Using a bubble jet printer print a grid of 20 strips onto a 18cm by 3cm section of nylon membrane (Magna nylon, MSI, Westboro MA or Nytran Supercharge, Schleicher and Schuell, Dassel GmbH, Germany).

(2) The vertical divisions between each strip should then be cut with a scalpel to avoid bleeding of the spots between strips.

(3) Approximately 0.3 microlitres of each oligonucleotide (1 mg/ml solution in water) should then be spotted onto the appropriate position on each strip (see Figure 1).

(4) Once dry, the membrane should be cross-linked by exposing to short wave UV in the Amplirad (GRI instruments UK) for 30 seconds.

(5) The membrane should then be washed 2 times in 50 ml of 0.5 x SSC for 2 minutes and air dried.

(6) The membrane can now be stored dry at room temperature ready for use.

PCR amplification

Add 1 microlitre of the DNA extract to 45 microlitres of the PCR mixture containing 0.5 microlitres of DnyaZyme (Flowgen) in a 200 microlitre PCR tube. A PCR negative control containing no bacterial DNA must be run alongside each set of PCR reactions.

In the PE thermal 2400 cycler (Perkin-Elmer Ltd.) carry out 5 cycles of 95° for 30 sec, 55°C for 15 sec, 72° C for 30 sec, followed by 25 cycles of 95°C for 15 sec, 65°C for 30 sec.

Hybridization

(1) Heat the PCR reactions to 95° C in the thermal cycler for 5 minutes.

(2) Label some hybridization strips and cut out with a scalpel and place in a screw-capped tube containing 0.5 ml of hybridization solution (5 x SSC, 0.01% SDS, 0.01 % N-laurylsarcosine, 1 % blocking reagent (Boehringer Mannheim Germany)).

(3) Pipette the PCR reactions into the appropriate tubes.

(4) Hold the hybridization reactions at 50° C for 45 minutes with gentle agitation.

Detection of hybridization

(1) Wash the strips 4 times in 25 ml of 0.25 x SSC + 0.1 % SDS at 37°C for 2 minutes.

(2) Flood the strips with 5 ml of blocking solution and leave for 15 minutes.

(3) Pour off the blocking solution and replace with 5 ml of maleic acid buffer containing 1 microlitre of the Anti-digoxigenin antibody conjugate (Boehringer Mannheim, Germany). Leave for 10 minutes.

(4) Wash the strips 4 times in 25 ml of maleic acid buffer for 1 minute.

(5) Flood the strips with detection buffer.

(6) Prepare 5 ml of the detection solution by adding 45 microlitres of BCIP and 35 microlitres of NBT to 5 ml of detection buffer.

(7) Pour off the detection buffer from the strips and replace with the detection solution prepared above.

(8) Leave the strips in the dark for 15 minutes then examine them for detectable hybridization. Record the results, after 45 minutes and terminate the development by washing the strips in distilled water.

The method of the present invention can be used to identify bacteria in settings other than those described above, both clinical and non-clinical, also in non-medical, agricultural and environmental applications e.g. testing water supplies, and in pure cultures after isolation. The method overcomes the problems of other similar molecular diagnostic techniques described above. It allows rapid diagnosis of such organisms in blood or blood cultures or in other clinical specimens such as cerebrospinal fluid, urine, joint fluid, swab specimens, and abscesses. It provides a set of universal primers and experimental conditions that can be used to amplify potentially characteristic sequences of bacterial 23S rDNA. In particular, it provides a series of specific oligonucleotide targets that can be used simultaneously in a hybridization assay for the identification of clinically important bacteria.

TABLE 1. Strains used in this study and results of PCR amplifications and hybridizations from culture.

Origin	Species	laboratory Code	Hybridization Strong	Weak
Blood culture STH	Staphylococcus epidermidis	36839	27, 7b, 8a	
Blood culture STH	Staphylococcus epidermidis	36938	27, 7b, 8a	
Blood culture STH	Staphylococcus epidermidis	44.3	27, 7b, 8a	
Blood culture STH	Staphylococcus epidermidis	37061	27, 7b, 8a	
Blood culture STH	Staphylococcus epidermidis	NCTC11047	27, 7b	
Blood culture STH	Staphylococcus warneri	B5	27, 7b	
Blood culture STH	Staphylococcus saprophyticus	B6	27, 7b	8a
Blood culture STH	Staphylococcus xylosus	B7	27, 7b	
Blood culture STH	Staphylococcus cohnii	B8	27, 7b	
Blood culture STH	Staphylococcus simulans	B9	27, 7b	
Blood culture STH	Staphylococcus hominis	B10	27, 7b	
Blood culture STH	Staphylococcus haemolyticus	B11	27, 7b	
Blood culture STH	Staphylococcus haemolyticus	31871	27, 7b	
NCTC	Staphylococcus aureus	NCTC6571	27, 7b 7a	
GH	Staphylococcus aureus (MR)	GH25	27, 7b 7a	
GH	Staphylococcus aureus (MR)	GH7	27, 7b 7a	
Blood culture STH	Staphylococcus aureus (MR)	816.98	27, 7b 7a	
Blood culture STH	Staphylococcus aureus (MS)	36989	27, 7b 7a	
Blood culture STH	Streptococcus milleri	676.98	27	
Blood culture STH	Streptococcus milleri	662.98	27	
Blood culture STH	Streptococcus pneumoniae	697.98	27, 5a	5b 7b 6a
Blood culture STH	Streptococcus pneumoniae	76a.98	27, 5a	7b
Blood culture STH	Streptococcus pneumoniae	736.98	27, 5a	7b
Blood culture STH	Streptococcus spp. (viridans)	738.98	27, 5a	7b
Blood culture STH	Streptococcus GroupG	776.98	27, 5a	
feces (VRE)	Enterococcus faecium	147	27, 6b	
feces (VRE)	Enterococcus faecium	152	27, 6b	
feces STH	Enterococcus faecium	7	27, 6b	
feces STH	Enterococcus faecium	24	27, 6b	
feces STH	Enterococcus faecium	39	27, 6b	
feces STH	Enterococcus faecium	40	27, 6b	
Blood culture STH	Enterococcus faecium	848.98	27, 6b	
Blood culture STH	Enterococcus faecium	665.98	27, 6b	
feces STH	Enterococcus faecalis	20	27, 5b	
feces STH	Enterococcus faecalis	23	27, 5b	
feces STH	Enterococcus faecalis	24	27, 5b	
feces STH	Enterococcus faecalis	25	27, 5b	
feces STH	Enterococcus faecalis	27	27, 5b	
feces STH	Enterococcus faecalis	82	27, 5b	
Blood culture STH	Enterococcus faecalis	707.98	27, 5b	
Blood culture STH	Enterococcus faecalis	706.98	27, 5b	
Blood culture STH	Enterococcus faecalis	708.98	27, 5b	
Blood culture STH	Enterococcus faecalis	835.98	27, 5b	
NCTC	Escherichia coli	NCTC8879	27, 3b	3a, 2b
Blood culture STH	Escherichia coli	817.98	27, 3b	3a, 2b
Blood culture STH	Escherichia coli	794.98	27, 3b	3a, 2b
Blood culture STH	Escherichia coli	829.98	27, 3b	3a, 2b
Blood culture STH	Escherichia coli	780.98	27, 3b	3a, 2b
Blood culture STH	Klebsiella oxytoca	800.98	27, 2a	3a, 2b
Blood culture STH	Klebsiella oxytoca	243a.95	27, 2a	3a, 2b
Blood culture STH	Klebsiella oxytoca	97.92	27, 2a	3a, 2b
Blood culture STH	Klebsiella pneumoniae	767.98	27, 2b	3a, 2b
Blood culture STH	Klebsiella pneumoniae	851.98	27, 2b	3a, 3b
Blood culture STH	Klebsiella pneumoniae	842.98	27, 2b	3a, 3b
Blood culture STH	Enterobacter cloacae	770.98	27, 3a	2b, 3b

Blood culture STH	Enterobacter cloacae		814.98	27, 3a	2b, 3b
Blood culture STH	Enterobacter cloacae	810.98		27, 3a	2b, 3b
Blood culture STH	Enterobacter aerogenes	743.98		27, 2b	3a, 3b
382010	Citrobacter freundii	382010		27, 2b, 3b	3a
Blood culture STH	Proteus mirabilis	827.98		27, 1a, 1b	
Blood culture STH	Proteus mirabilis	838.98		27, 1a, 1b	
Blood culture STH	Proteus mirabilis	703.98		27, 1a, 1b	
Blood culture STH	Serratia marcesens	1087.98		27, 2a, 2b, 3a, 3b	
Blood culture STH	Pseudomonas aeruginosa	37036		27, 4b	
Blood culture STH	Pseudomonas aeruginosa	812.98		27, 4b	
Blood culture STH	Pseudomonas aeruginosa	728.98		27, 4b	
Blood culture STH	Pseudomonas aeruginosa	714.98		27, 4b	
Blood culture STH	Pseudomonas aeruginosa	760.98		27, 4b	
Blood culture STH	Pseudomonas aeruginosa	702.98		27, 4b	
Blood culture STH	Pseudomonas aeruginosa	845.98		27, 4b	
Blood culture STH	Pseudomonas aeruginosa	37036		27, 4b	
Blood culture STH	Stenotrophomonas maltophilia		822.98	27, 4b	
Blood culture STH	Stenotrophomonas maltophilia		824.98	27, 4b	
CF patient LH	Burkholderia cepacia	H7		27, 4b	
CF patient LH	Burkholderia cepacia	F3		27, 4b	
CF patient LH	Burkholderia cepacia	TR1		27, 4b	
CF patient LH	Burkholderia cepacia	H9		27, 4b	
Blood culture STH	Coryneform	Co1		No Hybridization	
Blood culture STH	Coryneform	Co2		No Hybridization	
Blood culture STH	Candida albicans	C1		No amplicon or Hybridization	
Blood culture STH	Candida albicans	C2		No amplicon or Hybridization	

TABLE 1. Footnote
STH = St. Thomas' Hospital, GH = Guy's Hospital, LH = Lewisham Hospital, CF = Cystic fibrosis. NCTC = National Collection of Type Cultures, VRE = vancomycin resistant enterococci. MR = methicillin resistant, MS = methicillin senstitive

FIGURE 1 shows one very convenient pattern of oligonucleotide probes fixed to a supporting strip.

Sequence Listings for the primers and oligonucleotides used for the purposes of the present invention are given below.

CLAIMS

1. A method for identifying bacteria in a sample which comprises amplifying a portion of the 23S rDNA present in the sample using, as one primer, a degenerate primer set comprising one or more DNA molecules consisting essentially of DNA having the sequence(s)

5'GCGATTTCYGAAYGGGGRAACCC

the other primer consisting essentially of DNA having the sequence

5'TTCGCCTTTCCCTCACGGTACT

and testing the resulting amplicon by hybridisation to one or more oligonucleotide probes designed to identify one or more bacteria likely to be present in the sample.

2. Method according to claim 1, in which at least 8 bacterial species are tested for.

3. Method according to claim 2, in which the organisms tested for comprise at least one of Escherichia coli , Staphylococcus aureus , Pseudomonas aeruginosa , Enterococcus spp. , Klebsiella spp. , Enterobacter spp. , Proteus spp, Pneumococci, and coagulase negative Staphylococci.

4. Method according to claim 1, in which at least 10 bacterial species are tested for.

5. Method according to claim 4, in which the organisms tested for comprise at least one of Pseudomonas aeruginosa, Proteus mirabilis, Enterococcus feacium, Enterococcus feacalis , Staphylococcus aureus, coagulase negative Staphylococcus, Listeria species, Stenotrophomonas maltophilia , Burkholderia cepacia, and Escherichia coli.

6. A method according to claim 1, in which the oligonucleotide probe or probes has/have a sequence(s) selected from the group consisting of

SEQ ID Nos 3-7, 9-13, 15-19, 21-28, 30-32, 39-41, 44-49, 51, and 53-58.

7. A method according to claim 1, in which the oligonucleotide probe or probes has/have a sequence(s) selected from the group consisting of SEQ ID Nos 8, 14, 20, 29, 33-38, 42, 43, 50, 52, and 59.

8. A method according to claim 1, in which the oligonucleotide probe or probes has/have a sequence(s) selected from the group consisting of SEQ ID Nos 3-59.

9. A method according to claim 1, in which the oligonucleotide probe or probes has/have a sequence(s) selected from the group consisting of SEQ ID Nos 60 -63.

10. A method according to any of claims 1 to 9, in which amplification is carried out by the polymerase chain reaction (PCR)

11. A method according to any of claims 1 to 9, in which amplification is carried out by transcription mediated amplification.

12. A method according to any of the preceding claims, in which a plurality of oligonucleotide probes are used attached to a support material.

13. A degenerate primer set essentially comprising DNA having the sequences
5'GCGATTTCYGAAYGGGGRAACCC

14. A primer consisting essentially of DNA having the sequence
5'TTCGCCTTTCCCTCACGGTACT

15. A DNA sequence according to claim 13 or 14, being a labelled sequence.

16. A Digoxigenin-labelled DNA sequence according to claim 15,

17. One or more Oligonucleotides consisting essentially of one or more DNA molecules having sequences specified in claim 6.

18. One or more Oligonucleotides consisting essentially of one or more DNA molecules having sequences specified in claim 7.

19. One or more Oligonucleotides consisting essentially of one or more DNA molecules having sequences specified in claim 8.

20. One or more Oligonucleotides consisting essentially of one or more DNA molecules having sequences specified in claim 9.

21. One or more oligonucleotides according to any of claims 17 to 20, immobilised on a solid carrier.

22. A solid support material carrying one or more oligonucleotide probes as specified in claim 6, 7, 8, or 9.

23. A support material according to claim 22, in which some or all of the probes are attached to the substrate by means of chemically modified or additional bases.

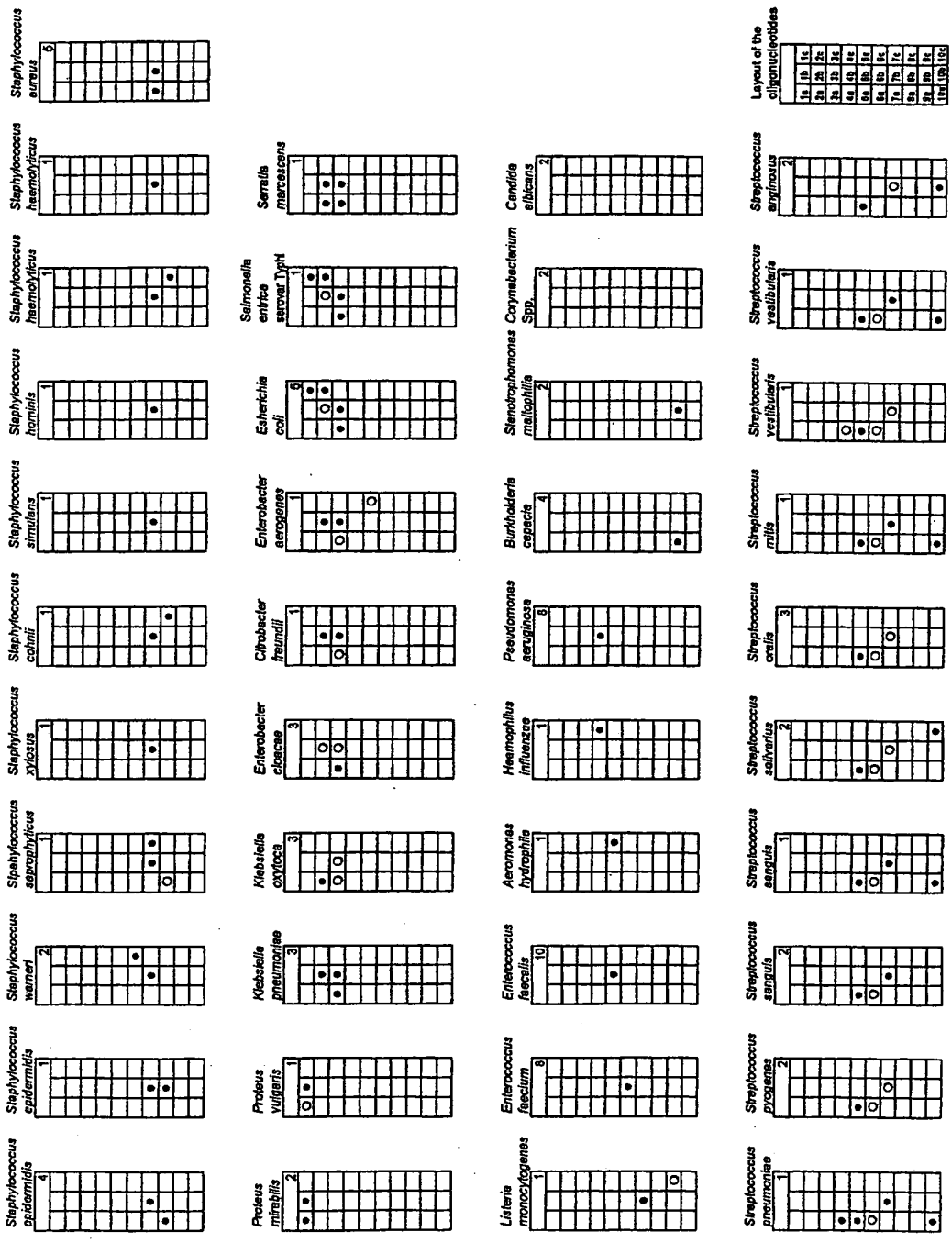
24. A support material according to claim 23, in which additional thymine bases have been attached to the 3 prime end of the probe to increase hybridization intensity.

25. A diagnostic kit for the identification of bacteria comprising one or more amplification primers specified in claim 1.

26. A diagnostic kit for the identification of bacteria comprising one or more oligonucleotide probes as specified in claim 6, 7 , 8, or 9.

27. A diagnostic kit for the identification of bacteria comprising a solid support material carrying one or more oligonucleotide probes as specified in claim 6, 7 , 8, or 9.

FIG. 1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Staphylococcus hominis
Staphylococcus simulans
Staphylococcus cohnii
Staphylococcus xylosus
Staphylococcus saprophyticus
Staphylococcus warneri
Staphylococcus epidermidis
Staphylococcus epidermidis
Proteus mirabilis
Listeria monocytogenes
Salmonella enterica serovar Typhi
Serratia marcescens
Escherichia coli
Enterobacter aerogenes
Citrobacter freundii
Enterobacter cloacae
Klebsiella oxytoca
Klebsiella pneumoniae
Proteus vulgaris
Enterococcus faecium
Enterococcus faecalis
Aeromonas hydrophila
Haemophilus influenzae
Pseudomonas aeruginosa
Burkholderia cepacia
Stenotrophomonas maltophilia
Corynebacterium Spp.
Candida albicans
Streptococcus anginosus
Streptococcus vestibularis
Streptococcus vestibularis
Streptococcus mitis
Streptococcus oralis
Streptococcus salivarius
Streptococcus sanguis
Streptococcus sanguis
Streptococcus pyogenes
Streptococcus pneumoniae
Layout of the oligonucleotides

SEQUENCE LISTING

<110> KING'S COLLEGE LONDON
GUY'S AND ST.THOMAS' NATIONAL HEALTH SERVICE TRUST
ANTHONY, RICHARD M
BROWN, TIMOTHY J
FRENCH, GARY L

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<130> N9557COPY

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